

Physiology of sporeforming bacteria associated with insects.

I. Glucose catabolism in vegetative cells

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Received October 30, 1969

BULLA, L. A., G. ST. JULIAN, R. A. RHODES, and C. W. HESSELTINE. 1970. Physiology of sporeforming bacteria associated with insects. I. Glucose catabolism in vegetative cells. *Can. J. Microbiol.* **16**: 243-248.

The catabolic pathways for use of glucose in proliferating vegetative cells of *Bacillus thuringiensis*, *B. alvei*, *B. lentimorbus*, and *B. popilliae* were studied by radiorespirometry. These organisms dissimilate glucose predominately via the Embden-Meyerhof-Parnas pathway and to a lesser extent by the pentose phosphate pathway. Extent of participation of concurrent pathways varied with each organism. Tentative evidence suggests that *B. popilliae* and *B. lentimorbus*, grown in a yeast extract-glucose medium, lack a fully operational tricarboxylic acid (TCA) cycle. Dilution of this medium slightly enhanced TCA cycle activity in *B. popilliae* but had no effect with *B. lentimorbus*. Radiorespirometric data regarding glutamic acid oxidation also were obtained for each bacterium. All organisms studied except *B. lentimorbus* were capable of oxidizing glutamic acid to carbon dioxide.

Introduction

Previous studies on carbohydrate metabolism in aerobic sporeforming bacteria have demonstrated that energy for vegetative growth is provided by oxidation of glucose (2, 6). The pathways by which glucose is oxidized in vegetative cells of several organisms also have been examined (3, 5, 8, 9, 12). These studies showed that glucose is dissimilated by the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PP) pathways.

Currently, we are investigating the metabolism of certain sporeforming bacteria associated with insect diseases. Our Laboratory has shown that large populations of *Bacillus popilliae* and *B. lentimorbus*, causative agents of milky disease in Japanese beetle larvae, can be grown readily in artificial culture and that such cells are both pathogenic and sporogenic when injected into larvae (14). However, *B. popilliae* sporulates only slightly in artificial culture (11, 15) and *B. lentimorbus* not at all. Spore formation is the key to use of these bacteria for biological control of Japanese beetles. Presumably, greater knowledge of their metabolism should provide a better understanding of the mechanism(s) for sporulation. To our knowledge, Pepper and Costilow (12, 13) have presented the only metabolic studies of these two organisms.

In this communication, results are presented of comparative biochemical studies with these

bacteria; *Bacillus thuringiensis*, producer of a toxin lethal to Lepidoptera larvae; and *Bacillus alvei*, a soil bacterium sometimes affiliated with European foulbrood in honey bees. The latter two organisms grow and sporulate under cultural conditions conducive only to vegetative growth of *B. popilliae* and *B. lentimorbus*. This work elucidates by a radiorespirometric method the pathways of glucose catabolism and the extent of their concurrent participation in proliferating vegetative cells of all four bacteria. Preliminary data on the oxidation of glutamic acid by each species and the effect of a change in medium concentration on simultaneous pathway participation in *B. lentimorbus* and *B. popilliae* also are presented.

Materials and Methods

Organisms and Cultural Conditions

The organisms studied were selected from the ARS Culture Collection: *B. thuringiensis* NRRL NRS-996, *B. alvei* NRRL B-384, *B. lentimorbus* NRRL B-2522, and *B. popilliae* NRRL B-2309.

Cultures of all organisms were maintained in liquid medium (designated JB) composed of 1.5% yeast extract, 0.6% K₂HPO₄, and 0.2% glucose. The medium, except for glucose, was dispensed into 300-ml Erlenmeyer flasks in 50-ml amounts and autoclaved at 15 p.s.i. for 15 min. Glucose was sterilized separately by membrane filtration and added aseptically before inoculation. Final pH of the medium was 7.4.

A 10% inoculum of mid-log phase cells previously transferred three times was used to obtain active growth. Flask cultures were aerated by rotary agitation at 250 rev/min at 28°C in an incubator shaker. Cells were harvested by centrifugation, washed twice with growth medium minus glucose, and then once with 0.05 M phosphate buffer (pH 7.4). After they were washed, cells were

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suspended in JB medium without glucose at a concentration of 1 mg/ml (dry weight) for radiorespirometric experiments.

Glucose Analysis

Glucose determinations were done by the *o*-toluidine method of Dubowski (7). This procedure allowed glucose to be measured in concentrations as low as 5 µg/ml.

¹⁴C-Labeled Substrates

Glucose-1-, 2-, 3-, 3,4-, and 6-¹⁴C, and uniformly labeled glutamic acid-¹⁴C were obtained from New England Nuclear Corp.,² Boston, Mass.

Radiorespirometric Technique

Radiorespirometric experiments, each done in triplicate, were performed according to the method of Wang (16). Incubation flasks, containing 30 mg of cell suspension, were placed in a modified Gilson differential respirometer at 28°C. Radioactive substrate (0.25 µcurie) was added from a sidearm on each flask; final volume was 30 ml. For glucose experiments, each flask contained 10 mg of specifically labeled glucose; for glutamic acid experiments, each flask contained 5 mg of uniformly labeled glutamic acid. Flasks were shaken while sparged with air at a rate of 60 cc/min. Endogenous metabolism was decreased by allowing the cultures to incubate for 30 min before addition of radioactive substrates. Respiratory ¹⁴CO₂ was trapped in 10 ml of a mixture of absolute ethyl alcohol and monoethanolamine (2:1, v/v), which was sampled and replaced hourly.

²Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Trapping solutions containing ¹⁴CO₂ were diluted to 15 ml with absolute ethyl alcohol. A 5-ml portion of each sample was placed in 10 ml of toluene containing 2,5-diphenyloxazole (6 mg/ml) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (100 mg/ml) in a 20-ml glass counting vial. Scintillation mixtures were counted by means of a Packard Tri-Carb model 2002 liquid scintillation spectrometer. All counting was done under balance point conditions and carried out to a standard deviation of no greater than 2%.

At the end of each experiment, the culture flasks were chilled and cells were separated from their incubation medium by centrifugation. Samples of cells and medium were mixed separately with Beckman solubilizer (Bio-Solv BBS-2), incubated at 25°C for 12 h, and placed in scintillation fluid for counting. Counting efficiency for each type of sample was determined by appropriate internal standards.

Calculation of Glucose-4-¹⁴C

Total recoveries obtained for glucose-4-¹⁴C were calculated from total recoveries of glucose-3- and -3,4-¹⁴C. The formula used for calculation of C-4 recovery data is $C-4 = 2(C-3,4) - (C-3)$. Curves for ¹⁴CO₂ from glucose-4-¹⁴C (Figs. 1-6), which show some irregularities, were plotted from hourly recoveries of the C-3 and C-3,4 glucose labels.

Results

Radiorespirometric patterns for *B. thuringiensis*, *B. alvei*, *B. lentimorbus*, and *B. popilliae* in JB medium when glucose was used as the major carbon source are presented in Figs. 1-4.

TABLE I
Isotope recoveries from catabolism of labeled glucose by four *Bacillus* species

| Organism | Glucose- ¹⁴ C | Percent isotope recoveries* | | | |
|-------------------------|--------------------------|-----------------------------|-------|--------|-------|
| | | CO ₂ | Cells | Medium | Total |
| <i>B. thuringiensis</i> | C-1 | 27 | 66 | 7 | 100 |
| | C-2 | 21 | 65 | 12 | 98 |
| | C-3 | 60 | 35 | 6 | 101 |
| | C-4 | 86 | 12 | 4 | 102 |
| | C-6 | 11 | 79 | 8 | 98 |
| <i>B. alvei</i> | C-1 | 25 | 64 | 9 | 98 |
| | C-2 | 33 | 46 | 14 | 93 |
| | C-3 | 46 | 42 | 11 | 99 |
| | C-4 | 61 | 30 | 10 | 101 |
| | C-6 | 19 | 64 | 16 | 99 |
| <i>B. lentimorbus</i> | C-1 | 16 | 69 | 9 | 94 |
| | C-2 | 4 | 58 | 37 | 99 |
| | C-3 | 44 | 33 | 12 | 89 |
| | C-4 | 74 | 22 | 3 | 99 |
| | C-6 | <1 | 47 | 54 | 101 |
| <i>B. popilliae</i> | C-1 | 26 | 62 | 9 | 97 |
| | C-2 | 9 | 55 | 32 | 96 |
| | C-3 | 62 | 30 | 7 | 99 |
| | C-4 | 64 | 30 | 8 | 102 |
| | C-6 | <1 | 44 | 54 | 98 |

*Averages of triplicate experiments.

Isotope recoveries from catabolism of labeled glucose in respired CO_2 , cells, and incubation media were obtained at the termination of each experiment (Table I).

The kinetics of catabolism of specifically labeled glucose by proliferating vegetative cells of all four organisms are typical of cells that use EMP as the major route and PP pathway as the minor for degradation of glucose (18). The differential rates of CO_2 evolution in the early period of incubation are $\text{C-4} > \text{C-3} > \text{C-1} > \text{C-2} > \text{C-6}$. This order agrees with the scheme

described by Wang and Krackov (17) for *B. subtilis*. In the EMP pathway glucose is converted to two molecules of pyruvate whose carboxyl groups correspond with carbons three and four of glucose. Subsequent oxidation produces C-4 and C-3 as CO_2 before the other carbons of pyruvate are oxidized. The rapid and extensive conversion of C-4 and C-3 to CO_2 clearly indicates the importance of the EMP pathway in these organisms.

Preferential oxidation to CO_2 of C-1 over C-6 and of C-4 over C-3 is evidence for operation of the PP pathway in these bacteria. In this pathway, glucose is converted to 6-phosphogluconate that is decarboxylated with the C-1 carbon,

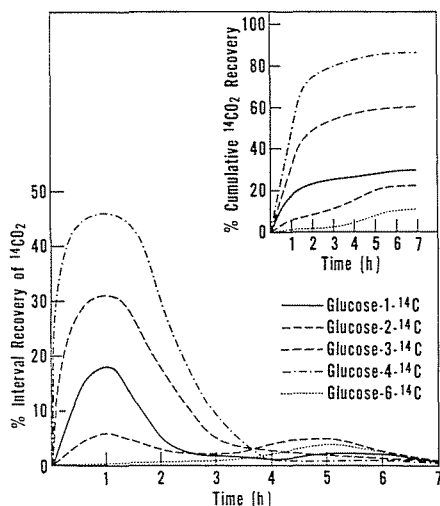


FIG. 1. Radiorespirometric patterns for use of glucose by *Bacillus thuringiensis* in JB medium (1.5% yeast extract, 0.6% K_2HPO_4). Ten milligrams of specifically labeled glucose (0.25 μcurie) was added to each reaction flask. Final volume was 30 ml.

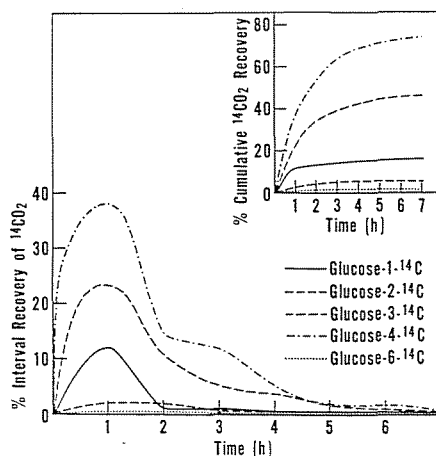


FIG. 3. Radiorespirometric patterns for use of glucose by *Bacillus lentimorbus* in JB medium.

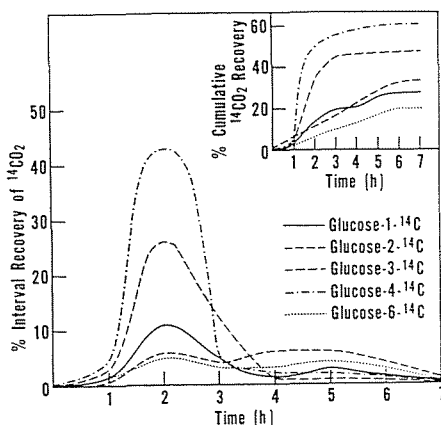


FIG. 2. Radiorespirometric patterns for use of glucose by *Bacillus alvei* in JB medium.

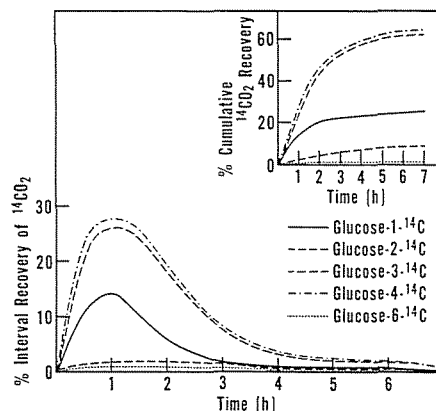


FIG. 4. Radiorespirometric patterns for use of glucose by *Bacillus popilliae* in JB medium.

appearing as CO_2 . The resulting pentose phosphate is either degraded to glyceraldehyde-3-phosphate or is recycled to form hexose phosphate, which, in turn, is catabolized concurrently by the EMP and PP pathways. Recovery of C-2 and C-6 as CO_2 from *B. thuringiensis* and *B. alvei* throughout the incubation period suggests extensive cycling of carbons via pentoses. Only slight activity occurred with *B. lentimorbus* and *B. popilliae*. We assume that redistribution of carbon atoms in re-formed hexose phosphate molecules follows the pattern described by Beevers (1).

Glucose was measured (see Materials and Methods) and found to be exhausted from the

TABLE II
Concurrent pathways of glucose catabolism in aerobically incubated vegetative cells of four *Bacillus* species*

| Organism | Percent pathway participation† | |
|-------------------------|--------------------------------|-------------------|
| | Embden-Meyerhof-Parnas | Pentose phosphate |
| <i>B. thuringiensis</i> | 84 | 16 |
| <i>B. alvei</i> | 94 | 6 |
| <i>B. lentimorbus</i> | 85 | 15 |
| <i>B. popilliae</i> | 75 | 25 |

*Medium contained 1.5% yeast extract, 0.6% K_2HPO_4 , and 0.2% glucose.

†Calculated according to method of Wang (16). Averages of triplicate experiments.

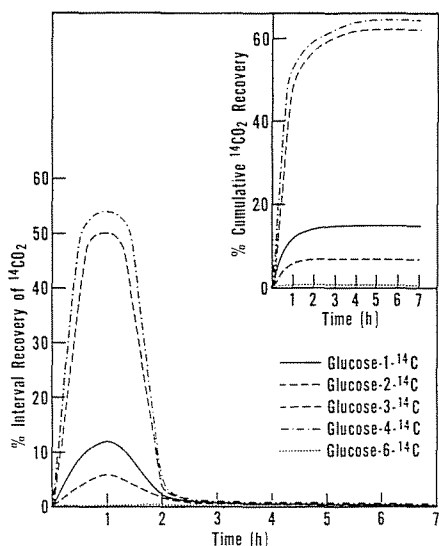


FIG. 5. Radiorespirometric patterns for use of glucose by *Bacillus lentimorbus* in dilute medium.

media by *B. thuringiensis* and *B. alvei* after $3\frac{1}{2}$ h incubation (Figs. 1 and 2) and after about 4 h by *B. lentimorbus* and *B. popilliae* (Figs. 3 and 4). Exhaustion of glucose marked the end of the assimilation phase. For *B. thuringiensis* and *B. alvei* a depletion phase followed in which the rates of CO_2 evolution are $\text{C-2} > \text{C-6} > \text{C-1} > \text{C-4}$ and C-3 . Intermediates from glucose catabolism were probably oxidized by tricarboxylic

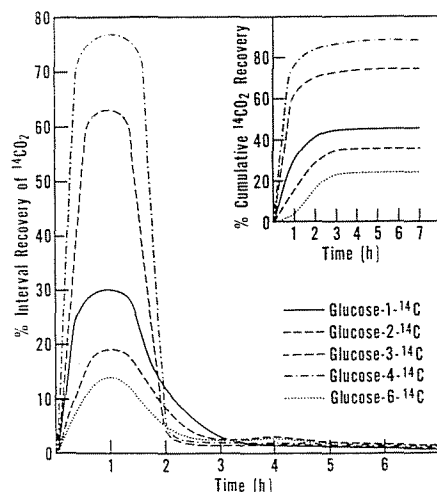


FIG. 6. Radiorespirometric patterns for use of glucose by *Bacillus popilliae* in dilute medium.

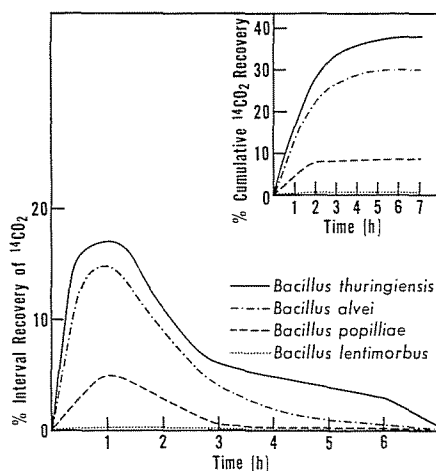


FIG. 7. Radiorespirometric patterns for use of DL-glutamic acid (uniformly labeled) by *Bacillus thuringiensis*, *B. alvei*, *B. popilliae*, *B. lentimorbus* in dilute medium (0.1% yeast extract and phosphate buffer). Five milligrams of uniformly labeled glutamic acid (0.25 μcurie) was added to each reaction flask. Final volume was 30 ml.

acid (TCA) cycle reactions during the depletion phase to give the observed order of CO₂ labeling. No depletion phase was observed in *B. lentimorbus* and *B. popilliae*. Apparently, intermediates of glucose catabolism either were concentrated within the cells or were used for biosynthetic purposes and not oxidized by the TCA cycle.

Total ¹⁴C recoveries from CO₂, cell incorporation, and medium as the percentage of total added glucose label (Table I) further substantiate the analyses of radiorespirometric data for all four organisms.

The radiorespirometric patterns for use of glucose are basically the same for all four bacteria, but the relative participation of concurrent pathways differs. As shown in Table II, use of glucose via the EMP pathway varied from 94% in *B. alvei* to 75% in *B. popilliae*. Pentose phosphate participation varied from 25% in *B. popilliae* to 6% in *B. alvei*. The values for *B. thuringiensis* and *B. lentimorbus* were intermediate.

Hanson *et al.* (10) have shown that vegetative cells of *B. cereus* grown in a complex medium similar to JB lack the enzymes necessary for terminal oxidation of acetate. However, such cells contain acetate-activating systems. With an exogenous supply of amino acids and related compounds in the JB medium, it is conceivable that certain TCA cycle enzymes of *B. lentimorbus* and *B. popilliae* were repressed. Therefore, attempts were made to derepress TCA cycle activity by incubating actively growing cells in a dilute medium consisting of 0.1% yeast extract and phosphate buffer. Data from these experiments are plotted in Figs. 5 and 6. As can be seen by comparison with Figs. 3 and 4, glucose was assimilated by both bacteria at a higher rate in dilute medium than in JB medium. Cumulative recoveries of C-4, C-3, and C-1 as CO₂ from *B. lentimorbus* were comparable in both media. For *B. popilliae*, recoveries of the same labels were markedly higher in dilute medium. Interestingly, C-2, but not C-6, was converted into CO₂ by *B. lentimorbus* and there was no depletion phase; appreciable quantities of both C-2 and C-6 were recovered as CO₂ from *B. popilliae* and a slight depletion phase was observed. Possibly, pentose cycling and TCA cycle reactions may have been derepressed in *B. popilliae* after medium dilution, whereas only pentose cycling was derepressed in *B. lentimorbus*.

Figure 7 depicts results of radiorespirometric experiments in which isotopically labeled glutamic acid is the major carbon source in dilute medium. These data indicate that *B. thuringiensis*, *B. alvei*, and *B. popilliae* oxidize glutamic acid, but that *B. lentimorbus* does not. A significant quantity of radioactive glutamate was measured in the latter two organisms to indicate its incorporation. Vegetative cells of *B. lentimorbus* may lack the enzymes of the catabolic and anabolic sequence of reactions in the TCA cycle and may use exogenous amino acids, such as glutamic acid, solely for biosynthetic purposes.

Discussion

Our studies demonstrate that proliferating vegetative cells of *B. thuringiensis*, *B. alvei*, *B. lentimorbus*, and *B. popilliae* catabolize glucose by concurrent operation of the EMP and PP pathways. Interestingly, recycling of pentoses seems to be extensive in *B. thuringiensis* and *B. alvei* but not in *B. lentimorbus* and *B. popilliae* under the same cultural conditions. The EMP pathway is the primary mechanism for glucose assimilation, whereas the PP pathway, by supplying reduced nicotinamide adenine dinucleotide phosphate, aids formation of biosynthetic intermediates rather than functioning as a major respiratory pathway.

TCA cycle activity was detected in *B. thuringiensis* and *B. alvei* but not in *B. lentimorbus* and *B. popilliae* when these organisms are grown in JB medium. Such results indicate that cultural conditions may affect terminal respiration in *B. lentimorbus* and *B. popilliae* differently than in *B. thuringiensis* and *B. alvei*. Activation of a TCA cycle is considered necessary for sporulation in aerobic sporeforming bacteria. Studies with *B. cereus* (10) and *B. subtilis* (3) indicate that this cycle provides the energy for biosynthesis and subsequent sporulation. Alternatively, some organisms sporulate with energy derived from oxidation of amino acids present in a medium similar to the one used in our experiments. Pepper and Costilow (12) suggested that failure of *B. popilliae* and *B. lentimorbus* to oxidize acetate may be reason for their inability to sporulate. We feel, as previously suggested (4), that biosynthetic events during the growth phase of *B. lentimorbus* and *B. popilliae* hamper activation of a TCA cycle; in turn, spore formation is excluded. Further studies are in progress

on the effect of nutritional conditions on the synthesis of key enzymes and intermediates of the EMP, PP, and TCA cycle pathways in these organisms.

Acknowledgments

We thank C. H. Wang, Oregon State University, and R. N. Costilow, Michigan State University, for their helpful advice during this investigation. Special appreciation is offered Gordon Adams of our Laboratory for his able technical assistance in this work.

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